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(71) Applicant *(for all designated States except US)*: ANTISOMA LIMITED [GB/GB]; Rochman Landau, Mappin House, 4 Winsley Street, London W1N 7AR (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : STUTTLE, Alan, William, John [GB/GB]; 24 Bourton Close, Hayes, Middlesex UB3 3PU (GB).

(74) Agents: LAMBERT, Hugh, Richmond et al.; D. Young & Co., 10 Staple Inn, London WC1V 7RD (GB).

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(54) Title: SYNTHETIC PEPTIDES FOR USE IN THROMBUS DETECTION

(57) Abstract

Radioactively labelled peptides comprising oligopeptides of from 3 to 10 peptide units and containing the sequence RGD and particularly the oligopeptides RGDSY and RGDFY, are disclosed as *in vivo* thrombus, tumour or CAM markers for the *in vivo* diagnosis and detection of thrombi, tumours or CAM in mammals.

* See back of page

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SYNTHETIC PEPTIDES FOR USE IN THROMBUS DETECTION

This invention relates to the development and use of synthetic peptides for thrombus detection both in human beings and animals, but primarily, of course, in the detection of human disease. The Method and the synthetic peptides used therein are also useful in targetting other sites in vivo, eg, cell adhesion molecules (CAMs) and tumors, containing an RGD binding site.

In 1984 Pierschbacher and Ruoslahti (Nature, 309, 30-33), showed evidence that the cell attachment activity of fibronectin could be mimicked by small synthetic peptide fragments. The amino acid sequence responsible for this activity was shown to be Arg-Gly-Asp-Ser (RGDS) and it was demonstrated that synthetic peptides containing this sequence were able to inhibit attachment of NRK cells (cells from a neuroblastoma cell line) to fibronectin coated substrates. The inhibition obtained with RGDS containing peptides was shown to be dose-related, whilst peptides which did not contain the RGDS sequence failed to inhibit cell attachment. The serine residue of the tetrapeptide has been shown to be non-essential, although only conservative substitutions may be made in order to retain biological activity.

The RGDS sequence has been shown to occur in fibrinogen, fibronectin and von Willebrand factor. Receptors for these proteins are expressed on the platelet membrane surface following platelet activation. Cross-linking of platelets via these cytoadhesive proteins accounts for the platelet-platelet interactions within a thrombus. It has also been demonstrated that RGDS containing synthetic peptides are capable of inhibiting platelet aggregation in vitro. This would suggest a specific interaction with the GP IIb/IIIa (glycoprotein fibrinogen receptor) complex present on the platelet membrane surface, which contains the fibrinogen binding domains. Extension of the RGDS sequence, by one amino acid residue at the carboxy and amino terminal, results in a ten-fold reduction in its biological activity, although further extension is not associated with a further reduction in binding capacity. Substitution of the serine residue by phenylalanine results in an anti-aggregatory peptide which is 4 to 5 times more potent than RGDS. There has also been suggestion that the residue corresponding to serine in the RGDS sequence may impart a degree of recognition specificity for

different RGDS receptors. This raises the possibility that both specificity and affinity could be modified by substitution around the RGD sequence. RGD binding sites are also known to occur on cell adhesion molecules (CAMs) and some tumors.

The present invention involves a novel approach to in vivo thrombus detection and which comprises the intravenous injection into the patient (which term herein includes both humans and animals, unless the context requires otherwise) of a radioactively labelled synthetic peptide having therein an RGD (Arg-Gly-Asp)-containing sequence, preferably an RGDS (Arg-Gly-Asp-Ser) or RGDF (Arg-Gly-Asp-Phe)-containing sequence having a specific binding affinity for the platelet GP IIb/IIIa complex, and detecting the presence, if present, of the bound label on the thrombus. Present methods of thrombus detection using labelled antibodies require several hours due to the slow rate of diffusion of the antibody through the system; using labelled peptides in accordance with the present invention is expected to enable thrombus detection in a matter of minutes, thus greatly facilitating diagnosis and treatment, and at a very early stage.

For use in that method of in vivo thrombus detection there is provided in accordance with the present invention a synthetic peptide containing the sequence RGD, preferably as RGDS or RGDF, and labelled with a radioactive label.

Suitable radioactive labels for use in the construction of such radioactively labelled peptides include: Tc^{99m}, I^{123} and In^{111}, and will be attached to the synthetic peptide in known manner, for example, via a cystine residue in the synthetic peptide. Other suitable techniques are described in Science, 220, 613-615; Int. J. Nucl. Med. Biol., 12, 3-8; J. Nucl. Med., 27, 27, 685-693 and J. Nucl. Med., 26, 293-299.

Subject to the dictates of suitability for parenteral administration and utility, i.e. high affinity and specificity for the GP IIb/IIIa complex, the precise amino acid sequence in terms of composition and length will not be particularly critical, although for practical reasons, e.g. economy and ease of synthesis, relatively short chain peptides will be preferred containing, for example, from 3 to 10 peptide units.

Suitable peptides containing an RGD sequence, preferably an RGDS or RGDF are available from a variety of different sources, or can be manufactured quite readily using conventional peptide synthesis procedures, and, in particular, using a conventional peptide synthesiser.

Also included within the scope of this invention are a diagnostic reagent for in vivo thrombus detection comprising a parenterally administrable solution of the radioactively labelled peptide containing an RGD sequence and a parenterally administrable carrier, and a method of in vivo thrombus detection which comprises intravenously administering a radioactively labelled peptide containing and RGD sequence capable of binding to RGD binding sites on platelets in the thrombus and radiographically detecting the accumulated bound peptide

The invention also extends to the use of the radioactively labelled peptides in in vivo localisation on to the RGD binding sites of CAMs.

Before proceeding further with the detailed description of this invention, and for the avoidance of doubt, the amino acid sequences referred to herein are identified by either their three letter abbreviations or single letter codes, as follows:

arginine	=	arg. or R.
aspartic acid	=	asp. or D.
glycine	=	gly. or G.
serine	=	ser. or S
tyrosine	=	tyr. or Y
phenylalanine	=	phe. or F
cysteine	=	cys. or C

Reference is also made hereinafter to the accompanying figure, which is a radiograph taken of a rabbit following intravenous administration of a radioactively labelled peptide according to this invention, and showing the localisation of the peptide in an artificially induced thrombus in the left ear.

Referring to th invention in slightly more detail, studies have been conducted using four peptides (RGDSY, RGDFY, RGDSYC and RGDSCRGDSY) to evaluate their potential as thrombus imaging agents.

The effect of these peptides on ADP-induced platelet aggregation was determined and compared with peptide RGDS which is known to inhibit platelet aggregation. The results (table 1) demonstrate that all four peptides studied are capable of inhibiting platelet aggregation at high concentrations and are virtually equipotent with RGDS. This suggests that inclusion of amino acids into these peptide sequences, to permit radio-labelling, does not destroy their ability to bind platelets (a prerequisite for thrombus imaging applications).

The second study involved radiodination of RGDSY, RGDFY, RGDSYC and RGDSCRGDSY with subsequent analysis of their ability to bind activated platelets in whole blood. The results (Table 2) indicate that all four peptides can bind platelets in ADP stimulated blood and that higher incorporation can be achieved in clotted blood.

One study was performed using RGDSY, labelled with the radioisotope iodine-123, injected into a rabbit who had a preformed thrombus in the microvasculature of the ear. The imaging studies, shown in the accompanying figure demonstrates a rapid uptake onto this thrombus (within 2 minutes of injection), which persisted for the period of study (20 minutes).

These data demonstrate that the four peptides studied are capable of binding to platelets, can be radiolabelled with gamma-emitting isotopes and are incorporated into platelet aggregates in stimulated and clotted blood. This provides good potential for thrombus detection and diagnosis by these peptides in vivo which has been confirmed, in an experimental animal model, using one of the peptides.

Table 1 Inhibition of ADP (1×10^{-5}M) -induced platelet aggregation by RGDS, RGDSY, RGDFY, RGDSYC and RGDSCRGDSY peptides.

(peptide)		percentage inhibition			
mM	RGDS	RGDSY	RGDFY	RGDSYC	RGDSCRGDSY
0.1	40/37	5/13	32	25	17
0.2	70/65	10/21	55	-	57
0.4	86/80	43/68	80	-	79

Table 2 Binding of radiolabelled RGDSY, RGDFY, RGDSYC and RGDSCRGDSY peptides ro ADP stimulated and clotted blood.

(peptide)	(bound peptide) ng			
ng	RGDSY	RGDFY	RGDSYC	RGDSCRGDSY
ADP Stimulated blood				
1	0.05	0.01	0.03	0.01
10	0.64	1.00	0.94	0.85
100	9.80	4.46	9.85	9.07
Clotted Blood				
1	0.27	0.41	0.18	0.28
10	0.85	2.14	2.26	2.64
100	17.27	18.12	27.08	29.33

The above results demonstrate the applicability of the invention over a range of synthetic peptides of different sizes all containing an RGD sequence. The actual length of the peptides is not critical, but for practical purposes the chain lengths may range from 3 to 10 peptide units, preferably 4 to 10 and, as already indicated, either consisting of or comprising an RGDS or RGDF sequence. Many such synthetic peptides are already available as known commercial products. Where not so available they can be readily synthesised by known peptide syntheses and/or using known peptide synthesisers. Similarlysaid synthetic peptides can be radioactively labelled by known techniques, for example, by iodination with I^{123} of a terminal tyrosine (Y) unit incorporated into the peptide.

The detailed pr paration of radioactively labelled peptides according to this invention is illustrated by the following example

Example

Preparation of radioactively labelled (I^{123}) RGDSY, RGDFY, RGDSYC and RGDSCRDSY

Iodogen tubes were prepared by dissolving Iodogen (1, 3, 4, 6-Tetrachloro-3α, 6α-diphenylglycouril) in chloroform at a concentration of $1mg.ml^{-1}$. Aliquots of 50µl (50µg Iodogen) were dispensed into polypropylene cryo-tubes and the chloroform evaporated to dryness. These tubes were then stored dessicated at -20°C until required.

Prior to radiolabelling the peptides were dissolved in phosphate buffered saline (PBS) at a concentration of $50\mu g.ml^{-1}$. RGDSYC and RGDSCRGDSY were first dissolved in a small volume of dimethyl sulphoxide (DMSO) such that the final concentration of DMSO in PBS was 1% v/v.

Iodogen tubes were equilibrated to room temperature before the addition of 200µl peptide solution and 1-10µl of ^{123}I (in aqueous solution). The reation mixture was then left for 15min at room temperature with occasional shaking. Following the incubation period the reaction mixture was removed and passed through a Sephadex G10 column which had been equilibrated with PBS. The column, which separates radiolabelled peptide from free iodine was eluted with PBS and 2ml fractions collected. Radioactivity in the fractions was measured and the eluted peptides, represented by the first radioactive peak from the column, collected and stored at 4°C until required.

The utility of the radioactively labelled peptides in in vivo thrombus detection is illustrated by the following experiment.

Experiment

Intravenous administsration of radioactively labelled (I^{123}) RGDSY to thrombitic rabbits

A male New Zealand White rabbit (3kg) was sedated by intramuscular injection of Hypnorm ($0.4mL.kg^{-1}$) and then anaesthetised by intravenous injection of Midazolam ($2mg.kg^{-1}$).

Two permanent disc magnets were positioned externally in the region of the jugular vein and the rabbit was then injected with 0.2g carbonyl iron microspheres suspended in 1ml of contrast media (Omnipaque) via an artery of the left ear. This procedure causes microthrombi in the capillary beds of the ear, whilst iron particles passing through the ear are trapped by the magnetic field and induce thrombus formation in the jugular vein. ^{123}I-RGDSY was injected intravenously into the contralateral ear 60min after injection of iron. Dynamic imaging by gamma camera was performed using a 1min frame rate for 20min with the camera positioned anteriorly to include both ears, head and neck regions in the field of view.

Following intravenous administration of the labelled peptide, the rabbit was radiographed and the resulting radiograph is presented in the accompanying figure. As indicated by the radiograph, there was rapid uptake of the peptide by a thromus in the jugular vein (arrow 1) and by multiple tiny thrombi in the left ear (arrow 2). The latter, in particular, demonstrates the possible utility of the invention in the detection of small thrombi in vivo and the possibility of early diagnosis and treatment.

CLAIMS

1 Radioactively labelled peptides containing from 3 to 10 peptide units capable of binding in vivo to an RGD binding site, and containing, in said 3 to 10 peptide units, the sequence arginine - glycine - aspartic acid (RGD).

2 Radioactively labelled peptides according to claim 1 consisting of or containing the sequence arginine - glycine - aspartic acid - serine (RGDS) or the sequence arginine - glycine - asapartic acid - phenylalanine (RGDF)

3 Radioactively labelled peptides according to claim 1 being specifically the peptides:
RGDSY, RGDFY, RGDSYC and RGDSCRGDSY

4. A radioactively-labelled peptide according to any one of claims 1 to 3, wherein the radioactive label is Tc^{99m}, I^{123} or In^{111}.

5. A diagnostic reagent for in vivo localisation on an RGD binding site comprising a parenterally administrable radioactively-labelled peptide containing an RGD sequence and a parenterally administrable carrier.

6 A diagnostic reagent according to claim 5, wherein said RGD binding site is a platelet binding site.

7 A diagnostic reagent according to claim 6, wherein said RGD binding site is a thrombus.

8 A diagnostic reagent according to claim 5, wherein said RGD binding site is of a cell adhesion molecule.

9. A diagnostic reagent according to claim 5, wherein said RGD binding site is on a tumor.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/00933

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 5/08, C 07 K 7/06, A 61 K 49/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0063002 (RESEARCH CORP.) 20 October 1982 see page 2, line 13 - page 3, line 13; page 18, table 1 --	1,2,5,7
Y	Biochemistry, volume 28, 4 April 1989, American Chemical Society, (Washington, US), J. Hawiger et al.: "Platelet receptor recognition domains on the ∝chain of human fibrinogen: structure-function analysis", pages 2909-2914 see pages 2910-2913, "Results and Discussion" --	1,2,5,7
A	Int. J. Radiat. Appl. Instrum., part B, Nucl. Med. Biol., volume 14, no. 3, 1987, Pergamon Journals Ltd, (Marsh Barton, Exeter, GB), F.L. Otsuka et al.: "Methods to label monoclonal antibodies for use in tumor imaging", pages 243-249 see page 244, column 2, "I-123"; ./.	4

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27th September 1990	18 OCT. 1990
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9000933 SA 38016

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/10/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0063002	20-10-82	US-A- 4427646	24-01-84
		AT-T- E11372	15-02-85
		CA-A- 1198671	31-12-85
		JP-A- 58013524	26-01-83
EP-A- 0333356	20-09-89	AU-A- 3098289	07-09-89
		WO-A- 9003391	05-04-90